**Plant totipotency and regeneration**

**Learning outcomes**

By the end of the practical you should:

* understand how differentiated plant cells can revert to an earlier developmental programme and redifferentiate into the complete range of other plant cells.
* observe the effect that morphogens (plant hormones) have on the developmental programme of differentiating cells.
* gain experience in plant tissue culture and aseptic technique.

**Introduction**

A plant is composed of several dozen different types of cells. Many of them can, in the right circumstances, differentiate into other cell types after becoming relatively undifferentiated, forming what is called **callus**, or even reprogramme into embryos, from which entire plants can form. In a way most plant cells can become reprogrammed into a "stem cell". The first step in reprogramming is generally the proliferation of cells in a de-differentiated state. This occurs naturally in response to **wounding**, as a form of repair: callus seals the wound. Rather than true “stem cells”, callus cells are in a “ground state” which is similar to root cortex or to leaf “parenchyma” without chloroplast differentiation. The ground state can be exploited in the lab. Reprogramming is triggered by chemical morphogens, plant hormones, auxins and cytokinins. Plant “stem cells” primarily occupy the growth apices, the shoot and root meristems, where new shoot with leaves or new root forms. Auxins and cytokinins play central roles in meristem formation and homeostasis, and can redirect differentiation towards either of these two programmes. This process is called **organogenesis**, and requires a number of cells (for example in the callus) becoming coordinated among themselves to form a new, functional meristem. If cells proliferate but meristem establishment and organogenesis fail, then callus tissue will continue to be produced. One reason for meristem establishment failure is the failure for gradients of auxin to form. One chemical, 2,4-D, has auxin action but cells cannot transport it (it is a **non-transportable auxin** analogue), so they cannot generate natural gradients with it. If cells are fully dispersed, small groups of cells formed from proliferation of individual ones can form what in effect is a new embryo, containing both shoot and root apical meristems. This process is termed **somatic embryogenesis** and can occasionally occur in callus.

In the practical you will use aseptically (sterile)-grown tobacco plants as the source of biological material. From this you will collect whole explants to place on different media with different balances of auxins and cytokinins. Each pair of you will test four media. After five weeks you will observe whether proliferation of undifferentiated tissue (callus) or differentiation (organogenesis) take place, and if they do, what new cell types or what kind of organ are formed.

**Organogenesis**

**Meristem**

Plant organ

**Callus**

**1. Initiation of a regeneration system from tobacco leaves**

**Dispersed cells**

**Somatic embryogenesis**

Whole plant

**Somatic embryogenesis**

Whole plant

Explant

(wounded tissue)

You will be provided with jars containing aseptically-grown tobacco plants, on an agar-solidified medium. The medium contains sucrose and other nutrients, and so it would become very quickly colonised by microorganisms (fungi) if exposed to the open air or any other such source. It is essential that all work be carried out in laminar-flow hoods (to which you will be directed), under sterile conditions. Follow the instructions for clean, safe work. Role your sleeves up. Wipe your hands or gloves with antiseptic solution. Avoid having items of clothing (including the sleeves of the lab coat) inside the flow hood work area. Flaming of tools is the key technique to maintain them sterile. Place your forceps or other tools in the ethanol container.

**Ethanol is very highly flammable.**

**Ensure that the ethanol container and the open flame are kept as far apart as possible, and that no spills occur.**

**When flaming a tool (scalpel, forceps), maintain it in a nearly horizontal, at a slightly downward angle, so that any remaining burning ethanol does not potentially run into your fingers.**

**Ensure that all manipulations are done cleanly, well into the hood, disturbing the laminar flow conditions minimally.**

* Open the jars containing the tobacco plants, and the plates with the four new media with different concentrations/types of auxins and cytokinins. Label appropriately.
* Take an explant of tobacco or a whole young plant onto a sterile Petri dish.
* Cut leaf explants using the scalpel against the Petri dish (don’t worry about damaging the dish). Hold the leaf in a way you do not excessively damage the eventual explant. Explants should be fragments of 5-10 mm in size, and with all edges cleanly cut if possible (to trigger the wounding response and to expose to nutrients and hormones through cut edges). Cut a few stem explants as well.
* Place two leaf fragments on each plate with the new medium. They should have the underside (paler-looking) facing up. Press the explants gently, so that as much as possible of the surface and edges are in contact with the medium. Put one stem explant also. Place the lid, and close it with parafilm. Label the plate base with the contents (leaf/stem, for each explant) and date and the top with your name(s).

These plates with different media will be cultured in a plant incubator for the coming five weeks.

The media composition is as follows, even though which which is unknown to you:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | AC | NTAC | HC | HA |
| Auxin | High | High, non-transportable | Low | High |
| Cytokinin | Medium/high | Medium/high | High | Low |

(Note “High” is here 5-8µM, medium 2-2.5µM, low 0.2-0.3µM)**2. Examination of callus and organogenesis outcomes.**

Take material out of each type of medium, and observe it in detail.

* Observe the leaf explants, particularly their boundaries. Record your observations. Amorphous, colourless tissue, appearing made of ‘bubbles’, would be callus. You will also see growth of a more defined shape; cut out a portion of that material, from its base, and observe whether organs looking like leaves are coming out individually from callus, or whether they are appearing in groups (as shoots with a leaf arrangement). Do the same for roots. When young, these would look like short white protrusions. If growing into the air, they will look fluffy because of visible root hairs.
* Examine also the stem explants. Note that any explant that contained the axil of a leaf would have contained a pre-existing (axillary) meristem. Growth from this one meristem is not due to hormone-induced organogenesis, but an axillary shoot. Still not all media may be equally appropriate for the survival of this axillary shoot.
* Estimate the number of points from which leaf-containing or root growth emerge for each plate, or the amount of callus. Use only the leaf explants. Give a relative numerical estimate, on a 0-10 scale, 10 corresponding to the plate with the largest amount of callus growth or the highest number of leaf-containng or root growth points. Put the information in this table:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Medium A | Medium B | Medium C | Medium D |
| Leaf-containing growth points |  |  |  |  |
| Root growth points |  |  |  |  |
| Callus relative amount |  |  |  |  |

* Cut if necessary small fragments, and bring them out to examine under a stereomicroscope.
* The demonstrator will select a representative example from each medium type, and post images for your lab report on moodle.

#### Questions to answer:

Note that you will be asked to address issues including the following:

* What was the effect caused by each of the culture media?
* The possible composition of the media (A, B, C, D) is given on the previous page (AC, NTAC, HC, HA). Can you speculate which is which, and why?

## Further sources

* Sablowski R (2009) Cytokinin and *WUSCHEL* tie the knot around plant stem cells. *Proc. Natl. Acad. Sci. USA* 103: 16016–16017\*. Pubmed id: [19805255](https://www.ncbi.nlm.nih.gov/pubmed/19805255)
* Greb T and Lohman JU (2016) Plant stem cells. *Current Biology* 26, R816–R821\* Pubmed id: [27623267](https://www.ncbi.nlm.nih.gov/pubmed/?term=greb+lohman+plant+stem+cells)

Also of use:

* <http://www.its.caltech.edu/~plantlab/research.html>